

EXHIBIT 2

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Brain 5-aminolaevulinate synthase

Developmental aspects and evidence for regulatory role

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1. Brain 5-aminolaevulinate synthase showed a peak of increased activity in the first few weeks of life, which preceded and accompanied the development of brain cytochromes. 2. In the brain of the adult rat the activity of the enzyme was only 20% of that in the liver (on a per g wet wt. basis), but it was still probably sufficient to maintain the turnover of brain cytochromes. 3. The brain synthase activity could be decreased by treatment of rats with cycloheximide or with large doses of 5-aminolaevulinate, especially when this precursor was given as the methyl ester. 4. Injected haematin and CoCl_2 markedly inhibited the synthase activity in the liver but failed to affect the brain enzyme; neither was taken up by the brain *in vivo*. 5. It is concluded that the brain can itself produce the haem required for the synthesis and turnover of its own haemoproteins and that 5-aminolaevulinate synthase may regulate the pathway in brain as in other tissues. 6. The relevance of the present findings to the pathogenesis of the neurological symptoms of acute porphyria and to the beneficial effect of exogenous haematin in porphyric patients is briefly considered.

Acute intermittent porphyria is an inborn error of metabolism characterized by a partial block in liver haem biosynthesis and by increased urinary excretion of the intermediates of the pathway, 5-aminolaevulinate and porphobilinogen. During the phases of exacerbation of the disease, or acute attacks, the excretion of 5-aminolaevulinate and porphobilinogen becomes even greater and a number of symptoms may appear, all attributable to impairment of nervous function. Thus, although the liver appears to be the main site where the intermediates of the pathway are produced in excess, the clinical symptoms pertain almost exclusively to the nervous system.

There are two main hypotheses for the pathogenesis of the neurological symptoms of the acute attack (Shanley *et al.*, 1977; Meyer & Schmid, 1974). (1) The excessive amounts of porphyrin precursors made in the liver may lead to a build up within the nervous system of a toxic principle responsible for the neurological symptoms. (2) The partial block of haem biosynthesis due to the genetic

defect may be expressed in the nervous tissue (as well as in the liver) and lead there to a decreased supply of haem, which may then in turn cause impaired metabolism and loss of function of the neurons. Large doses of exogenous haem are said to be beneficial when given to a porphyric patient during an acute attack (Watson *et al.*, 1973; Jeelani Dhar *et al.*, 1975; Watson, 1975; Peterson *et al.*, 1976). This beneficial effect could be explained by either of the two hypothetical mechanisms considered above: exogenous haem is known to inhibit formation of the intermediates of the pathway in the liver (by lowering the activity of hepatic 5-aminolaevulinate synthase; Bonkowsky *et al.*, 1971), and could in this way prevent the formation of the postulated neurotoxic principle; also, if haem were taken up by the brain in sufficient amounts, it could conceivably rectify a state of haem deficiency in the nervous system.

The work described in the present paper was stimulated by our interest in the biochemical mechanism of the nervous manifestations of porphyria and was aimed at answering two basic questions. (1) Is the brain capable of producing haem for its own needs, even under conditions where haem requirements are increased, or does it depend

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on other organs and tissues (for example on the liver; Bissell *et al.*, 1979; Maines, 1980) for its haem requirement? (2) Can exogenously administered haem be taken up by the brain *in vivo*?

Materials and methods

Animals

Male Sprague-Dawley rats were allowed food and water *ad lib.* and were adjusted to a 12h light/12h darkness cycle for at least 1 week before killing. With the exception of rats given cycloheximide, CoCl_2 , haematin or 5-aminolaevulinate *in vivo*, where individual rats were studied, the livers and brains from two animals were pooled for each observation. The effect of cycloheximide and CoCl_2 was also studied in male Porton rats with very similar results. Animals were killed by decapitation (between 11:00h and 13:00h) and their brain and liver were obtained within 30s and transferred into an ice-cold homogenizing fluid containing sucrose (0.25M), Tris/HCl buffer, pH 7.2 (10mM) and EDTA (1mM). The whole brain was taken for homogenization, including the cerebellum.

Methaemalbumin was prepared (Tenhunen *et al.*, 1968), using bovine serum albumin; [^{14}C]haemin (sp. radioactivity 0.1 Ci/mol) was prepared biosynthetically from 5-aminol[4- ^{14}C]laevulinate. 5-Aminolaevulinic acid and its methyl ester were injected intravenously dissolved in a propylene glycol/saline (0.9% NaCl) mixture (1:2, v/v) and CoCl_2 and cycloheximide dissolved in physiological saline. Doses and time of killing are given in the Results section.

Studies *in vitro*

5-Aminolaevulinate synthase (EC 2.3.1.37) was assayed by a slight modification of the radiochemical method described by Condie & Tephly (1978). The incubation mixture contained in a total volume of 2ml the following components with final concentrations, or total amounts, in parentheses: liver or brain homogenate (equivalent to 100mg wet wt. of tissue), Tris/HCl buffer (75mM), glycine (100mM), EDTA (7.5mM), pyridoxal 5'-phosphate (0.4mM), ATP (1.36mM) and [2,3- ^{14}C]succinate (sp. radioactivity 2.73 Ci/mol; 0.37mM). The pH of the complete mixture was 7.5 at 20°C. Incubation was for 1h at 38°C in the presence of air but without shaking and zero-time samples and incubated blanks (containing no enzyme) were routinely run. Under these conditions conversion of [2,3- ^{14}C]succinate into 5-aminolaevulinate was linear with time of incubation (up to at least 1h) and with amount of tissue taken (up to at least 100mg). When the concentration of succinate was increased to 3.6mM, only 12–25% greater yields of 5-aminolaevulinate were obtained. Since this entailed a considerable loss

of sensitivity and much greater amounts of labelled succinate were therefore required, the concentration of succinate was kept at 0.37mM throughout the present work. In some experiments conversion of [1,4- ^{14}C]succinate was also studied.

5-Aminolaevulinate was obtained by ion-exchange chromatography and further purified by treatment with acetylacetone to form the pyrrole derivative 3-acetyl-4-(2-carboxyethyl)-2-methylpyrrole (Mauzerall & Granick, 1956) and by extracting the pyrrole into ethyl acetate at pH 4.6 (Condie & Tephly, 1978). Overall recovery of 5-aminolaevulinate, determined by taking through the whole procedure a known amount of 5-aminol[^{14}C]laevulinate was in excess of 90%. The radioactive compound recovered in the ethyl acetate phase was compared with the pyrrole obtained from authentic 5-aminol[^{14}C]laevulinate by determining (1) the ratio between the radioactivities recovered in the first and second ethyl acetate extracts of the eluate, (2) the ratio between the radioactivities present in the ethyl acetate before and after shaking it with an excess of unlabelled eluate of pH 4.6 and (3) the recovery of radioactivity from silica gel 60 plates (0.25mm thick; E. Merck, Darmstadt, Germany), after t.l.c. in butanol/acetic acid (Mauzerall & Granick, 1956) and visual detection of the pyrrole spot by spraying with a mixture of modified Ehrlich/mercury reagent (Urata & Granick, 1963) and methanol (1:4, v/v). By all these criteria the radioactivity recovered in the ethyl acetate behaved as that obtained from authentic 5-aminolaevulinate and no significant contamination of the ethyl acetate extract by other radioactive compounds could be detected.

Portions (1 ml) of the ethyl acetate extract were taken for counting radioactivity in a TriCarb model B 2450 liquid-scintillation spectrometer with Insta-gel as the scintillant, and correction for quench was by an external-standard/channel-ratio curve. Yields of 5-aminolaevulinate were calculated from the specific radioactivity of the [^{14}C]succinate at a 1:1 molar ratio. The radioactivities of blood plasma and of tissue homogenates from animals injected with [^{14}C]haematin were similarly determined and the contribution of blood plasma to the brain and liver radioactivity was calculated by assuming a 45% haematocrit value and a blood plasma content of 1.7% (Chiueh *et al.*, 1978) and of 7.7% (Cunningham *et al.*, 1976), for brain and liver respectively.

Source of special chemicals

Cycloheximide, bovine serum albumin (essentially fatty acid-free) and 5-aminolaevulinic acid (both the unesterified acid and its methyl ester) were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. 1,2-Propylene glycol (purum grade) was from Kebo AB, Stockholm, Sweden, and haemin samples

were from Universitätslabor für Medizinische Chemie, C. J. Wiemann, U activity radioactivity laevulinate obtained Dreieiche

Results

Conversion of 5-aminolaevulinate

In the present work we used brain haem into 5-aminolaevulinate by determining (1) the ratio between the radioactivities recovered in the first and second ethyl acetate extracts of the eluate, (2) the ratio between the radioactivities present in the ethyl acetate before and after shaking it with an excess of unlabelled eluate of pH 4.6 and (3) the recovery of radioactivity from silica gel 60 plates (0.25mm thick; E. Merck, Darmstadt, Germany), after t.l.c. in butanol/acetic acid (Mauzerall & Granick, 1956) and visual detection of the pyrrole spot by spraying with a mixture of modified Ehrlich/mercury reagent (Urata & Granick, 1963) and methanol (1:4, v/v). By all these criteria the radioactivity recovered in the ethyl acetate behaved as that obtained from authentic 5-aminolaevulinate and no significant contamination of the ethyl acetate extract by other radioactive compounds could be detected.

When we converted a 15–40% carbon and brain poration

Table 1. Conversion of 5-aminolaevulinate to 5-aminolaevulinate. Brain radioactivity was calculated by assuming a 45% haematocrit value and a blood plasma content of 1.7% (Chiueh *et al.*, 1978) and of 7.7% (Cunningham *et al.*, 1976), for brain and liver respectively.

Labelled succinate [2,3- ^{14}C] [1,4- ^{14}C]

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obtained by ion- further purified by o form the pyrrole rethyl)-2-methylpyr- 6) and by extracting : pH 4.6 (Condie & very of 5-amino- g through the whole of 5-amino[¹⁴C]- %. The radioactive l acetate phase was ned from authentic mining (1) the ratio red in the first and the eluate, (2) the present in the ethyl it with an excess of (3) the recovery of) plates (0.25 mm any), after t.l.c. in & Granick, 1956) e spot by spraying h/mercury reagent ethanol (1:4, v/v). ty recovered in the ined from authen- gnificant contamin- ct by other radio- id.

etate extract were i a TriCarb model ymeter with Insta- m for quench was atio curve. Yields ulated from the succinate at a 1:1 blood plasma and als injected with ermined and the re brain and liver assuming a 45% lasma content of of 7.7% (Cun- rain and liver

umin (essentially ic acid (both the r) were obtained ., London S.W.6, grade) was from haemin samples

were generous gifts from Dr. S. Sassa, Rockefeller University, New York, NY, U.S.A., and from Dr. C. J. Watson, Northwestern Hospital, Minneapolis, MN, U.S.A. [1,4-¹⁴C]Succinic acid (sp. radioactivity 51.3 Ci/mol), [2,3-¹⁴C]succinic acid (sp. radioactivity 68.1 Ci/mol) and 5-amino[5-¹⁴C]-laevulinic acid (sp. radioactivity 49 Ci/mol) were obtained from New England Nuclear G.m.b.H., Dreieichenhain, Germany.

Results

Conversion of [¹⁴C]succinate into 5-aminolaevulinate and into unknown metabolite *in vitro*

In the first experiment, in which adult male rats were used, evidence was obtained that both liver and brain homogenates could convert [¹⁴C]succinate into 5-aminolaevulinate *in vitro*, liver being considerably more active than brain (Table 1). This confirms previous findings (Paterniti *et al.*, 1978; Percy & Shanley, 1979; Maines, 1980). When the trichloroacetic acid-soluble supernatants from incubation of either tissue with [2,3-¹⁴C]succinate were subjected to ion-exchange chromatography, the 1.0M-sodium acetate eluates contained, in addition to 5-aminolaevulinate, some other unknown metabolite(s). The amount of radioactivity present as unknown metabolite was relatively small with liver but very marked with brain, where it exceeded the radioactivity present as 5-aminolaevulinate by 50-fold. In spite of this large excess, complete separation of 5-aminolaevulinate from the unknown contaminant was achieved by a further purification step, as described in the Materials and methods section.

When [2,3-¹⁴C]succinate and [1,4-¹⁴C]succinate were compared as precursors of 5-aminolaevulinate, a 15–40% greater incorporation of the methylene carbon atoms of succinate was found with both liver and brain (Table 1), compared with the incorporation of the carboxy carbon atoms of succinate.

Table 1. Conversion of [¹⁴C]succinate into 5-aminolaevulinate by brain and liver homogenates *in vitro*: comparison between [2,3-¹⁴C]- and [1,4-¹⁴C]-succinate. Brain and liver homogenates were incubated as described in the Materials and methods section with either [2,3-¹⁴C]- or [1,4-¹⁴C]-succinate (0.37 mM). Results are those of individual observations, each obtained with the liver and brain from a different adult rat and are given as pmol of [¹⁴C]-succinate converted into 5-aminolaevulinate/min per g wet wt. of tissue.

Labelled succinate	Succinate converted (pmol)	
	Brain	Liver
[2,3- ¹⁴ C]	25, 25, 32	132, 145, 111, 134
[1,4- ¹⁴ C]	15, 21, 21	116, 131, 88, 108

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Postnatal development of 5-aminolaevulinate synthase in brain and liver

Previous work has shown an age-dependent maturation of several metabolic pathways in the brain, including the respiratory activity of brain (Land *et al.*, 1977) and its concentration of mitochondrial cytochromes (Chapelinsky & Arnaiz, 1970; Bull *et al.*, 1979). All of these components or activities are very low at birth, then steadily increase reaching the adult activity at approx. 20 days of age. In contrast, the activity of 5-aminolaevulinate synthase, which was also very low at birth, showed a

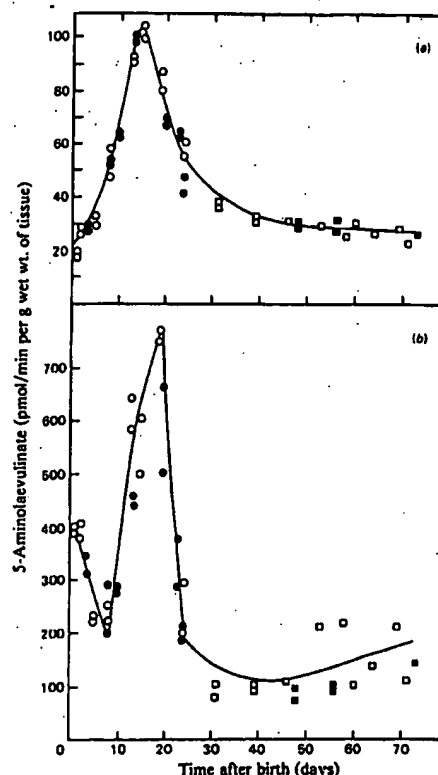


Fig. 1. Postnatal development of brain and liver 5-aminolaevulinate synthase in the rat

Male rats were killed at different times after birth and the conversion of [2,3-¹⁴C]succinate into 5-aminolaevulinate by their brain (a) and liver (b) homogenates was determined. Each point represents the result obtained with the pooled homogenates from two rats. Different symbols represent batches of rats with a slightly different birth date. Note that the scales of the ordinates differ in (a) and (b).

rapid increase thereafter, reaching a peak at about 15 days and subsequently declining to the much lower adult value (Fig. 1a). Therefore there was a sharp peak of 5-aminolaevulinate synthase activity preceding and accompanying the increase in mitochondrial cytochromes, suggesting a physiological response to the increased haem demand.

The age-dependent behaviour of 5-aminolaevulinate synthase was somewhat different in rat liver, where activities at birth were relatively high and a peak of increased activity was noted (at about 20 days of age), after an initial decline (Fig. 1b). Both the initial decline in hepatic 5-aminolaevulinate synthase activity in the first few days after birth and the peak of increased activity at around 20 days of age have already been reported (Woods & Dixon, 1970; Maines & Kappas, 1978). In the liver, turnover of microsomal cytochromes, especially cytochrome P-450, accounts for most of the haem that is synthesized (Meyer & Schmid, 1977); this is in contrast with the situation in the brain, where most of the newly formed haem appears to be destined for the synthesis of mitochondrial cytochromes (Percy & Shanley, 1979). It is therefore noteworthy that in the liver the wave of

increased 5-aminolaevulinate synthase (Fig. 1b) again precedes and accompanies the maturation (Song *et al.*, 1971) of the predominant haemoproteins, in this case the cytochromes of the P-450 group.

Effect of various treatments on brain and liver activities of 5-aminolaevulinate synthase in adult rats

The finding of increased brain 5-aminolaevulinate synthase at a time during development when haem requirements might be expected to be greater suggested that in brain, as in other organs (for example, the liver), this enzyme might regulate haem synthesis and be subjected to homeostatic control. Several treatments were compared for their effect on the brain and liver enzymes. It was found (Table 2) that cycloheximide decreased the enzyme activity in both tissues, whereas intravenously administered haem (given in either of two different forms) or subcutaneously injected CoCl_2 decreased the enzyme activity only in the liver. Cycloheximide, CoCl_2 and haematin have already been reported to prevent the stimulation of hepatic 5-aminolaevu-

Table 2. Effect of administering to rats either cycloheximide, CoCl_2 , haematin or 5-aminolaevulinic acid on the conversion *in vitro* of [2,3- ^{14}C]succinate into 5-aminolaevulinate by their brain and liver homogenates

Male rats (240–320 g) were given various treatments by subcutaneous (s.c.) or intravenous (i.v.) injections as indicated and were killed 1 h or 2 h later. Control rats were injected with saline, albumin, Na_2CO_3 or propylene glycol solutions as appropriate. Conversion of [2,3- ^{14}C]succinate into 5-aminolaevulinate was measured *in vitro* as described in the Materials and methods section. Results given are means \pm S.E.M. of at least four observations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, when compared with corresponding controls of the same experiment, but injected with solvent alone. Further abbreviation used: N.D., not detectable in any of the four samples.

Experiment	Treatment and dose	Time of killing (h after treatment)	[2,3- ^{14}C]Succinate converted into 5-aminolaevulinate (pmol/min per g wet wt. of tissue)	
			Brain	Liver
A	Saline (10 ml/kg body wt., s.c.)	1	31.1 \pm 1.8	137.4 \pm 15
	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (60 mg/kg body wt., s.c.)	1	32 \pm 1.4	28.1 \pm 9***
	Cycloheximide (40 mg/kg body wt., i.v.)	1	21.1 \pm 2**	61.2 \pm 10*
B	Albumin (1.25%, in saline) (0.8 ml/kg body wt., i.v.)	2	35 \pm 0.41	129 \pm 39
	Methaemalbumin (equivalent to 3.2 mg of haemin/kg body wt., i.v.)	2	38.5 \pm 4.6	12.5 \pm 2.4*
C	Na_2CO_3 (0.25%) (1.8 ml/kg body wt., i.v.)	2	33.9 \pm 2.4	109 \pm 24.4
	Haematin in Na_2CO_3 solution (equivalent to 7.5 mg of haemin/kg body wt., i.v.)	2	31.9 \pm 1.9	11.3 \pm 1.6**
D	Propylene glycol/saline (1:2, v/v) (0.8 ml/kg body wt., i.v.)	1	35.5 \pm 1.5	72.7 \pm 12
		2	33 \pm 2.5	75 \pm 16
	5-Aminolaevulinic acid (methyl ester) in propylene glycol/saline (22.8 mg/kg body wt., i.v.)	1	18.1 \pm 1.4***	6.1 \pm 1**
		2	14.7 \pm 1.9**	3.1 \pm 1**
	5-Aminolaevulinic acid (free acid) in propylene glycol/saline (21.2 mg/kg body wt., i.v.)	2	32.5 \pm 1.3	N.D.

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Table 3. Re

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5-aminolaevulinate opment when haem ted to be greater other organs (for might regulate haem omoestatic control. ed for their effect on was found (Table 2) e enzyme activity in ously administered different forms) or, decreased the en- ver. Cycloheximide, dy been reported to patic 5-aminolaevu-

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Liver	
137.4 ± 15	
28.1 ± 9***	
61.2 ± 10*	
129 ± 39	
12.5 ± 2.4*	
109 ± 24.4	
11.3 ± 1.6**	
72.7 ± 12	
75 ± 16	
*** 6.1 ± 1**	
** 3.1 ± 1**	
N.D.	

Table 3. Radioactivity recovered at different times after injection of [¹⁴C]haematin in blood plasma and homogenates of brain, liver, kidney and spleen of the rat
Male rats were injected intravenously with [¹⁴C]haematin and killed at different times thereafter. In experiment (a) rats (body wt. 129–139 g) were injected with methaemalbumin (equivalent to 4.9–5.3 mg of haemin/kg body wt.) and in experiment (b) rats weighed 278–284 g and were given haematin dissolved in 0.25% Na₂CO₃ at a dose of 6.7–7.4 mg of haemin/kg body wt. Results given refer to individual rats.

Experiment	Tissue	Radioactivity recovered at 30 min after injection (d.p.m./g or d.p.m./ml)		Time course of radioactivity (% of that observed at 3 min)			
		Observed	Corrected for entrapped plasma	3 min.	15 min	30 min	90 min
(a)	Plasma	5885	—	100	58.8	29.4	16.5
	Brain	116	16	100	58.0	35.3	18.5
	Liver	3707	3254	100	190.2	187.0	185.8
	Kidney	1041	—	100	82.8	85.5	68.2
	Spleen	725	—	100	126.7	97.2	82.5
(b)	Plasma	16737	—	100	—	66.0	39.3
	Brain	309	25	100	—	67.7	44.4
	Liver	7175	5886	100	—	351.0	420.4
	Kidney	1348	—	100	—	99.3	114.0
	Spleen	1533	—	100	—	114.9	155.0

lininate synthase caused by inducers (Hayashi *et al.*, 1969; Gayathri *et al.*, 1973; Maines *et al.*, 1976; De Matteis & Gibbs, 1977) and CoCl₂ also to cause a decrease in the basal activity of the liver enzyme (Nakamura *et al.*, 1975; Sinclair *et al.*, 1979).

An intravenous load with 5-aminolaevulinate did not affect markedly the brain synthase (even though it completely abolished the enzyme activity in the liver), but injection of similar amounts of the methyl ester of 5-aminolaevulinic acid caused a very significant decrease in the activity of the brain enzyme (Table 2). Previous studies have shown that parenterally injected 5-aminolaevulinic acid is taken up with difficulty by the brain but very effectively by the liver (McGillion *et al.*, 1975; Shanley *et al.*, 1976), and we suggest that the marked inhibition of the brain enzyme observed after a load of the methyl ester reflects passive diffusion of the unchanged methyl ester across the blood/brain barrier, followed by hydrolysis to the free amino acid within the brain and subsequent conversion of 5-aminolaevulinate into haem. Uptake of several amino acid esters (but not of the corresponding free amino acids) has also been reported with rat liver lysosomal preparations incubated *in vitro* and the methyl esters have been shown to undergo hydrolysis to the free amino acids within the lysosomes (Reeves, 1979).

The differential effect of Co²⁺ on 5-aminolaevulinate synthase in the two organs is also probably due to differential uptake, as the concentration of cobalt was found to be considerably higher in liver (13.5 µg/g wet wt. of tissue, on average) than in brain (<0.3 µg/g wet wt.) of treated animals. Evidence is provided below that the lack of effect of intravenously administered haematin on brain 5-

aminolaevulinate synthase was similarly due to lack of uptake by brain tissue.

Fate of intravenously administered labelled haematin

Rats were injected with [¹⁴C]haematin, either as methaemalbumin or as a solution in 0.25% (w/v) Na₂CO₃. The latter was also used as several investigators have claimed that haematin administered in this form is beneficial to patients suffering from the neurological symptoms of acute porphyria. No evidence for brain uptake of haematin could be obtained. For example, when methaemalbumin was injected (Table 3a), in agreement with previous findings (Snyder & Schmid, 1965), the liver radioactivity was in excess of that expected from contamination by entrapped plasma, but the radioactivity recovered in brain was very small and most of it could be accounted for by contaminating blood plasma. In addition, the time course of radioactivity suggested substantial uptake of label by liver (and possibly to some extent by kidney and spleen), whereas the time course of the brain radioactivity followed very closely that of plasma, again suggesting that the small amount of label present in brain is probably due to its blood plasma content. Similar findings were obtained with haematin dissolved in Na₂CO₃ solution (Table 3b).

Discussion

We have confirmed the presence in the brain of the first enzyme of the pathway of haem biosynthesis, 5-aminolaevulinate synthase, and also found that the enzyme undergoes changes in activity

at different stages of development from birth to adult age. The wave of increased activity seen in the first few weeks of life preceded and accompanied the formation of brain haemoproteins, suggesting a physiological response of the brain enzyme to ensure adequate supply of haem within the brain cells during a period of increased demand. This suggests that the brain can itself produce the haem required for the synthesis of its own haemoproteins and that it accomplishes this, like other organs, by increasing the activity of the rate-controlling enzyme of haem biosynthesis, 5-aminolaevulinate synthase.

In the adult rat both the concentration of the main haemoproteins of the brain (the mitochondrial cytochromes; see Bull *et al.*, 1979) and the activity of brain 5-aminolaevulinate synthase (Fig. 1a) are approximately in a steady state. The amount of haem, and therefore of 5-aminolaevulinate, required to maintain the turnover of brain cytochromes was calculated, using the total cytochrome content given by Bull *et al.* (1979) (46.8 nmol/g wet wt. of cortical slices) and assuming the same ϵ value (132h), which has been reported (Aschenbrenner *et al.*, 1970) for the turnover of mitochondrial cytochromes in both liver and heart of the adult rat. The amount of 5-aminolaevulinate required (33 pmol/min per g of brain) is in good agreement with activity of 5-aminolaevulinate synthase observed in this work between 46 and 70 days of age (29 ± 0.8 pmol/min per g (mean \pm S.E.M.); Fig. 1a). This also suggests that the brain is capable of producing haem for its own needs and is not likely to depend on the liver (Maines, 1980; Bissell *et al.*, 1979) for its haem requirements.

This work has also shown that the brain synthase activity can be decreased, like the liver enzyme, by giving cycloheximide or by large doses of 5-aminolaevulinate, especially when this precursor of haem is administered as the methyl ester, presumably because it can then diffuse more readily across the blood/brain barrier and generate intercerebrally enough haem to exercise a feedback type of action on the enzyme. Paterniti *et al.* (1978) and Percy & Shanley (1979) have reported that brain 5-aminolaevulinate synthase is not affected by administration of either ethanol, 2-allyl-2-isopropylacetamide or 3,5-diethoxycarbonyl-1,4-dihydrocollidine, treatments all known to induce the liver enzyme, suggesting some basic difference in the way in which the enzyme is regulated in the two organs. Percy & Shanley (1979) have further suggested that an inability of brain 5-aminolaevulinate synthase to respond with induction may explain the neurological symptoms of acute porphyria, as the inherited enzymic block in the pathway of haem biosynthesis may not be compensated in the brain (as it is in the liver) by increased production of the haem precursors. Our observations would on the

other hand suggest that brain 5-aminolaevulinate synthase may be regulated by haem as in other tissues and that the failure of inducers of the liver enzyme to stimulate the synthase in brain is due to them all acting indirectly, for example by interaction with the drug-metabolizing system (De Matteis, 1978), the activity of which is known to be very low in brain (Sasame *et al.*, 1977). More direct evidence that the brain 5-aminolaevulinate synthase is in fact inducible in the adult rat has recently been found (F. De Matteis & D. E. Ray, unpublished work).

Previous work (Shemin, 1955) has shown that succinate can be converted into 5-aminolaevulinate by two different pathways. In the first pathway succinyl-CoA, the immediate precursor of 5-aminolaevulinate, is generated directly by the enzyme succinyl-CoA synthetase (EC 6.2.1.5); in the second, succinate enters the tricarboxylic acid cycle and succinyl-CoA is generated from the oxidative decarboxylation of α -oxoglutarate. Since in the latter pathway both carboxy carbon atoms of succinate are lost, whereas both methylene carbon atoms are retained, incorporation of [1,4- 14 C]succinate into 5-aminolaevulinate can only take place by the succinyl-CoA synthetase pathway. In contrast, incorporation of radioactivity from [2,3- 14 C]-succinate will proceed by both pathways (Shemin, 1955). It can therefore be concluded from the results of Table 1 that both pathways operate in the brain (as they do in the erythropoietic system (Shemin, 1955) and in the liver (see also Granick & Urata, 1963)) and that under the standard conditions of our enzymic assay most of the succinate is utilized for 5-aminolaevulinate synthesis, via the succinyl-CoA synthetase pathway.

When radioactive [14 C]haematin was administered intravenously to rats, uptake of radioactivity by liver (and possibly by kidney and spleen) could be demonstrated, but no significant uptake was found in the case of brain. This indicates that the blood/brain barrier is impermeable to haematin and casts doubts on the hypothesis that exogenous haem might remedy a situation of brain haem deficiency during the acute attack of human porphyria. If haematin injections are in fact beneficial to these patients, they are more likely to act by suppressing excess formation of 5-aminolaevulinate in the liver and its subsequent conversion into the postulated neurotoxic principle.

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55) has shown that 5-aminolaevulinate is the first pathway precursor of 5-aminolaevulinate by the enzyme 5-aminolaevulinate synthase (EC 2.3.1.5); in the carboxylic acid cycle from the oxidative state. Since in the latter atoms of succinate the carbon atoms are 4-¹⁴C)succinate into take place by the hway. In contrast, y from [2,3-¹⁴C]- pathways (Shemin, ded from the results operate in the brain etic system (Shemin, o Granick & Urata, lard conditions of our cinate is utilized for ia the succinyl-CoA

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References

- Aschenbrenner, V., Druyan, R., Albin, R. & Rabinowitz, M. (1970) *Biochem. J.* 119, 157-160
- Bissell, D. M., Liem, H. H. & Muller-Eberhard, U. (1979) *Biochem. J.* 184, 689-694
- Bonkowski, H. L., Tschudy, D. P., Collins, A., Doherty, J., Bossenmaier, I., Cardinal, R. & Watson, C. J. (1971) *Proc. Natl. Acad. Sci. U.S.A.* 68, 2725-2729
- Bull, R. J., Lutkenhoff, S. D., McCarty, G. E. & Miller, R. G. (1979) *Neuropharmacology* 18, 83-92
- Chepelinsky, A. B. & Arnaiz, G. R. D. L. (1970) *Biochim. Biophys. Acta* 197, 321-323
- Chiueh, C. C., Sun, C. L., Kopin, I. J., Fredericks, W. R. & Rapoport, S. I. (1978) *Brain Res.* 145, 291-301
- Condie, L. W. & Tephly, T. R. (1978) *Methods Enzymol.* 52, 350-354
- Cunningham, V. J., De Matteis, F. & Stonard, M. D. (1976) *Biochem. J.* 158, 105-108
- De Matteis, F. (1978) in *Heme and Hemoproteins* (De Matteis, F. & Aldridge, W. N., eds.), pp. 129-155. Springer-Verlag, Berlin
- De Matteis, F. & Gibbs, A. H. (1977) *Biochem. J.* 162, 213-216
- Gayathri, A. K., Rao, M. R. S. & Pudmanaban, G. (1973) *Arch. Biochem. Biophys.* 155, 299-306
- Granick, S. & Urata, G. (1963) *J. Biol. Chem.* 238, 821-827
- Hayashi, N., Yoda, B. & Kikuchi, G. (1969) *Arch. Biochem. Biophys.* 131, 83-91
- Jeelani Dhar, G., Bossenmaier, I., Petryka, Z. J., Cardinal, R. & Watson, C. J. (1975) *Ann. Int. Med.* 83, 20-30
- Land, J. M., Booth, R. F. G., Berger, R. & Clark, J. B. (1977) *Biochem. J.* 164, 339-348
- Maines, M. D. (1980) *Biochem. J.* 190, 315-321
- Maines, M. D. & Kappas, A. (1978) *J. Biol. Chem.* 253, 2321-2326
- Maines, M. D., Janousek, V., Tomio, J. M. & Kappas, A. (1976) *Proc. Natl. Acad. Sci. U.S.A.* 73, 1499-1503
- Mauzerall, D. & Granick, S. (1956) *J. Biol. Chem.* 219, 435-446
- McGillion, F. B., Thompson, G. G. & Goldberg, A. (1975) *Biochem. Pharmacol.* 24, 299-301
- Meyer, U. A. & Schmid, R. (1974) *Res. Publ. Assoc. Res. Nerv. Ment. Dis.* 53, 211-223
- Meyer, U. A. & Schmid, R. (1977) in *The Metabolic Basis of Inherited Disease* (Stanburg, J. B., Wyngaarden, J. B. & Frederickson, D. S., eds.), pp. 1166-1220. McGraw-Hill, New York
- Nakamura, M., Yasukochi, Y. & Minakami, S. (1975) *J. Biochem. (Tokyo)* 78, 373-380
- Paterniti, J. R., Simone, J. J. & Beattie, D. S. (1978) *Arch. Biochem. Biophys.* 189, 86-91
- Percy, V. A. & Shanley, B. C. (1979) *J. Neurochem.* 33, 1267-1274
- Peterson, A., Bossenmaier, I., Cardinal, R. & Watson, C. J. (1976) *J. Am. Med. Assoc.* 235, 520-522
- Reeves, J. P. (1979) *J. Biol. Chem.* 254, 8914-8921
- Sasame, H. A., Ames, M. M. & Nelson, S. D. (1977) *Biochem. Biophys. Res. Commun.* 78, 919-926
- Shanley, B. C., Percy, V. A. & Neethling, A. C. (1976) in *Porphyrias in Human Diseases* (Doss, M., ed.), pp. 155-162. Karger, Basel
- Shanley, B. C., Percy, V. A. & Neethling, A. C. (1977) *S. Afr. Med. J.* 51, 458-460
- Shemin, D. (1955) *Harvey Lect.*, 258-284
- Sinclair, P., Gibbs, A. H., Sinclair, J. F. & De Matteis, F. (1979) *Biochem. J.* 178, 529-538
- Snyder, A. L. & Schmid, R. (1965) *J. Lab. Clin. Med.* 65, 817-824
- Song, C. S., Moses, H. L., Rosenthal A. S., Gelb, N. A. & Kappas, A. (1971) *J. Exp. Med.* 134, 1349-1371
- Tenhunen, R., Marver, H. S. & Schmid, R. (1968) *Proc. Natl. Acad. Sci. U.S.A.* 61, 748-755
- Urata, G. & Granick, S. (1963) *J. Biol. Chem.* 238, 811-820
- Watson, C. J. (1975) *N. Engl. J. Med.* 293, 605-607
- Watson, C. J., Jeelani Dhar, G., Bossenmaier, I., Cardinal, R. & Petryka, Z. J. (1973) *Ann. Int. Med.* 79, 80-83
- Woods, J. S. & Dixon, R. L. (1970) *Life Sci.* 9, 711-719

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